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Human-derived, plant-produced monoclonal antibody for the treatment of anthrax

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Abstract

The unpredictable nature of bio-terrorism compels us to develop medical countermeasures that will enable authorities to treat individuals exposed to agents such as anthrax. We report the feasibility of producing a protective, human-derived, monoclonal antibody directed against the protective antigen (PA) of *Bacillus anthracis* in plants. This was achieved by transient expression using agroinfiltration of *Nicotiana benthamiana* plants. The resulting antibody was able to neutralize toxin activity in vitro and in vivo at a comparable level to that seen for its hybridoma-produced counterpart.

Keywords: Anthrax; Antibody; Plant

1. Introduction

In a bio-terrorism attack the aggressor is likely to strike at a time and place calculated to induce maximum terror through mass casualties. The absence of specific intelligence and integrated real-time detection systems coupled with the unpredictable nature of such events necessitates the development of medical countermeasures that will enable authorities to rapidly treat exposed individuals. The credentials of *Bacillus anthracis* as a bioterror agent were confirmed by the 2001 postal attacks in the USA. Following inhalation, anthrax spores are phagocytosed by alveolar macrophages and transported to hilar and tracheobronchial lymph nodes where the spores germinate, and multiplication of vegetative bacilli occurs [1,2]. Fatal bacteraemia and toxaemia then ensue, with a mortality rate in untreated individuals of >80% [3].

Early treatment is essential. Animal studies suggest that as the disease progresses due to the accumulation of a lethal level of toxin antibiotics become ineffective [4]. *B. anthracis* produces a tripartite toxin consisting of two enzymatically active subunits, lethal factor (LF) and edema factor (EF) that combine with protective antigen (PA), the non-toxic cell-binding component, to form lethal toxin (LT) and edema toxin, respectively [5]. Given the central role of anthrax toxin in pathology of the disease, the ability to neutralize its effects would be of immense value at all stages of the infection.

Several animal studies have demonstrated that anthrax toxin-specific antibodies from vaccinated animals can passively protect recipients [6–8]. Horse serum has also been used to treat people exposed to anthrax [9]. However, the drawbacks inherent in using animal-derived sera (serum sickness) have prompted the adoption of alternative approaches such as the use of anthrax immunoglobulin derived from immunized donors [10]. While this approach offers a number of advantages such as reduced adverse reactions, prolonged serum half life, and the targeting of multiple epitopes, it does suffer from the costly need to maintain, and constantly re-

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new adequate stocks of quality-controlled and safety-tested antibodies with sufficiently high toxin neutralization titer to protect a large numbers of individuals. An alternative approach would be to develop toxin-neutralizing human monoclonal antibodies (mAbs) with specificity for either PA or LF, or both [11]. In conjunction, improved mAb production systems will be needed that are capable of generating safe, efficacious antibodies at a scale and cost which will support large scale stockpiling necessary for rapid response to an attack.

Plant-based antibody production systems have the capacity to meet this need as they offer specific advantages such as product safety, low cost and ease of scale-up compared to conventional fermentation methods [12]. Plants have proven to be effective systems for producing functional therapeutic mAbs capable of conferring passive protection against bacterial and viral pathogens in animals [13].

We report here the production and evaluation of a plant-produced PA-specific toxin-neutralizing mAb (pp-mAb^{PA}) derived from a human immunized with a licensed anthrax vaccine. The resulting antibody was found to be as efficacious as its hybridoma-produced counterpart. This approach offers the potential to develop urgently needed medical countermeasures at a fraction of the cost of traditional cell culture-based approaches.

2. Materials and methods

2.1. DNA constructs

The *Sfi*I site at position 11031 of pBI121 (Accession number AF485783) was mutagenized followed by introduction of new *Sfi*I sites in positions 5825 and 7711 to create pBISfi.

Total RNA was isolated from the anti-PA mAb-expressing hybridoma line IQ6E4 (IQ Corporation, The Netherlands) using RNeasy Mini Kit (Qiagen, Valencia, CA). Heavy and light chain coding sequences were amplified by RT-PCR using Superscript One-Step RT-PCR kit with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The heavy chain (HC) was amplified using the degenerate 5′-primer, 5′-CTCGCGGCCCAGCCGGCCATGGACTGSAYCTGGAG-3′, in combination with the constant region 3′-primer 5′-CTCGCGGCCTCCGAGGCCTCATTTACCCKGAGACAGG-3′. The light chain (LC) was amplified using a mixture of three 5′ primers 5′-CTCGCGGCCCAGCCGGCCATGGACATGAGGGTCCYCGC-3′, 5′-CTCGCGGCCCAGCCGGCCATGAGGSTCCYTGCTCAGCT-3′, 5′-CTCGCGGCCCAGCCGGCCATGGAARCCCCAGCGCAGCT-3′ in combination with the constant region 3′-primer 5′-CTCGCGGCCTCCGAGGCCCTAACACTCTCCCCTGTTGA-3′. Resulting HC and LC fragments were cloned in pBISfi to create pBISfi- IQ6E4G (encoding HC) and pBISfi- IQ6E4K (encoding HC).

2.2. Transient gene expression

Agrobacterium tumefaciens strain GLA 4404 (Invitrogen, Carlsbad, CA) was transformed with pBISfi-IQ6E4G and pBISfi-IQ6E4K. *Agrobacterium* cultures were grown and induced as described [14]. Equal volumes of the induced suspensions (each at OD_{600}2.4) were mixed and used in agroinfiltration of *Nicotiana benthamiana* leaves. The leaves were harvested 2.5 days post-infiltration and stored at −80 °C.

2.3. Antibody purification

The plant tissue was homogenized in extraction buffer (50 mM Tris–HCl, pH 7.5, 10 mM sodium diethyldithiocarbamate and Complete Mini protease inhibitors (Roche Applied Science, Indianapolis, IN)) with 1% PVPP (polyvinylpolypyrrolidone). The extract was incubated for 1 h at 4 °C with 0.1% Tween®20. Plant debris was removed by filtration through Miracloth (Calbiochem, San Diego, CA) followed by centrifugation. The antibody was recovered using ammonium sulfate precipitation and HiTrap protein A column chromatography (Amersham, Piscataway, NJ). The pp-mAb^{PA} was further purified by thiophilic adsorption chromatography using T-gel adsorbent (Pierce, Rockford, IL). Hybridoma-produced monoclonal antibody (hp-mAb^{PA}), was purified similarly using protein A and T-gel chromatography.

2.4. ELISA and Western blot analysis

Antibody concentrations were estimated by ELISA using Nunc Maxisorb plates (Fisher Scientific, Hampton, NH). pp-mAb^{PA} and hp-mAb^{PA} were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Whole human IgG (Jackson Immunoresearch Laboratories) was used as a standard. Specificity of pp-mAb^{PA} was determined by ELISA and Western blotting. For ELISA, plates were coated with 0.5 μg of PA/well (Alpha Diagnostic International, San Antonio, TX). In Western blot analysis and ELISA pp-mAb^{PA} bound to PA was detected by HRP-conjugated rabbit anti-human IgG.

2.5. Toxin neutralization assay

Toxin-neutralizing activity was determined using a murine macrophage/monocyte (RAW 264.7) cell line culture and a WST-1-based cell viability quantitation assay (Roche Molecular Biochemicals, Indianapolis, IN). Serial dilutions of PA-specific antibodies were assayed against standard concentrations of LF and PA. RAW 264.7 cells (TIB-71, American Type Culture Collection, Manassas, VA) were plated at 5×10^4 cells/well in a microtiter plate in D-MEM supplemented with 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD), 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 10 mM HEPES buffer solution (Gibco

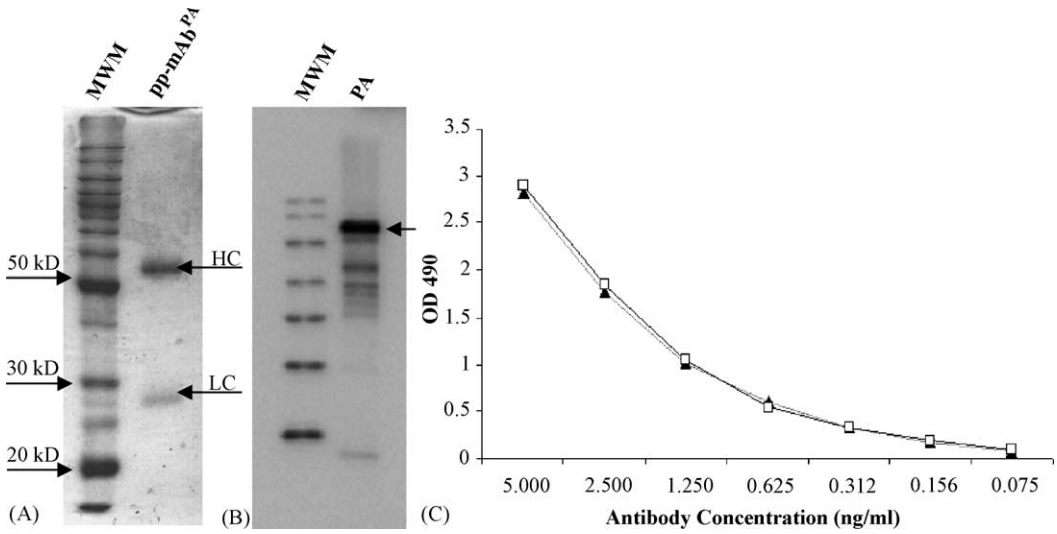

Fig. 1. Purification and characterization of pp-mAb^{PA}. (A) Coomassie staining of purified pp-mAb^{PA}. (B) Binding of pp-mAb^{PA} to PA (→) in Western blot. (C) Binding of pp-mAb^{PA} (▲) and hp-mAb^{PA} (□) to PA in ELISA. MWM indicate molecular weight markers.

BRL), Gentamicin (25 μg/ml), and 1 mM sodium pyruvate (Gibco BRL) approximately 4 h prior to the assay. All incubations were at 37 °C and 5% CO_2 atmosphere, 95% relative humidity. Test antibodies were prepared in a separate 96-well microtiter plate as two-fold dilutions in four or five replicas and incubated with 100 ng/ml PA at 37 °C for 30 min. PA (pre-incubated with or without antibody) and LF were added to the cells (respective concentrations 100 ng/ml and 50 ng/ml) and incubated for 12–15 h. WST-1 was added and cell viability measured using an ELISA reader (OD_{450}).

2.6. Animal protection studies

The ability of the pp-mAb^{PA} and hp-mAb^{PA} to protect A/J mice against challenge with spores of the Sterne strain of *B. anthracis* was determined according to the method of Beedham and colleagues [8]. A group of five mice were given 180 μg of pp-mAb^{PA} by the intra-peritoneal route in PBS. Control mice received either the equivalent concentration of the hp-mAb^{PA} or PBS. 2.5 h after passive immunization animals received spores of *B. anthracis*, at a dose of 1×10^4 spores in 0.1 ml of PBS (approx 30 median lethal doses). Following challenge, animals were monitored daily for 14 days for evidence of morbidity or mortality.

3. Results

3.1. Characterization of antibody expression

cDNAs for HC and LC of hp-mAb^{PA} were cloned from the IQ6E4 hybridoma cell line and transiently expressed from the *Cauliflower Mosaic Virus* 35S RNA promoter by agroinfiltration in *N. benthamiana* plants. The pp-mAb^{PA} was purified by protein A affinity chromatography followed by thiophilic adsorbent chromatography. SDS-PAGE analysis demonstrated high purity and integrity of the pp-mAb^{PA} (Fig. 1A). The yield of purified antibody was approximately 1 mg/kg plant tissue.

The hybridoma line producing hp-mAb^{PA} was selected based on its ability to bind PA and to neutralize LT. The specificity of pp-mAb^{PA} for PA was confirmed by Western blotting (Fig. 1B) and in ELISA (Fig. 1C). The pp-mAb^{PA} efficiently bound to PA as determined by Western blotting and was indistinguishable from hp-mAb^{PA} in its performance in ELISA. This result indicates that production in plants did not impair the specificity of antibody for PA.

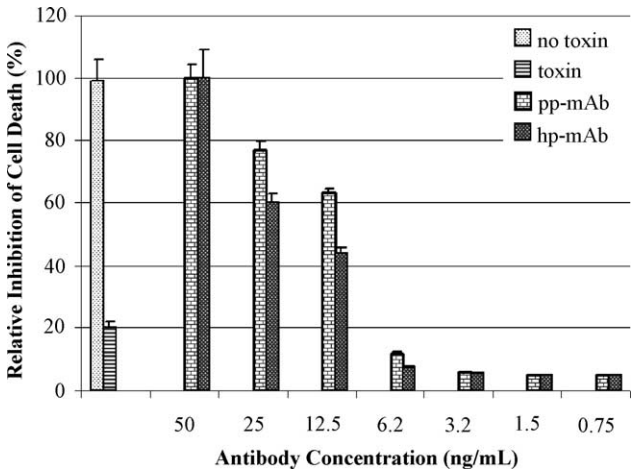

Fig. 2. Percentage of relative inhibition of cell death as result of toxin neutralization by pp-mAb^{PA} or hp-mAb^{PA}. mAbs were assessed at different concentrations for the ability to neutralize the lethal toxin and prevent cell death. No toxin is a control representing RAW 264.7 cells grown without any toxin or mAbs. Toxin indicates RAW 264.7 cells grown in the presence of PA and LF. The data points represent the average from five independent replicates.

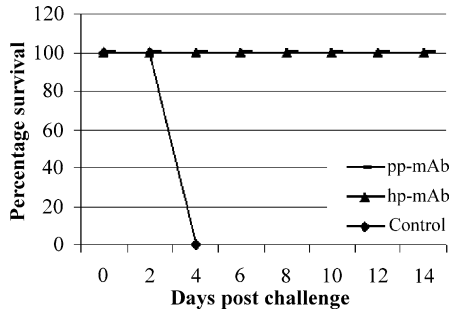

Fig. 3. Animal protection studies. 2.5 h after receiving a single dose (180 μg) of pp-mAb^{PA} A/J mice were exposed to lethal dose of anthrax spores. Control mice received either equivalent quantity of hp-mAb^{PA} or PBS. Animals receiving PBS developed clinical signs of infection and succumbed to disease 3 days after challenge. Animals receiving pp-mAb^{PA} or hp-mAb^{PA} did not develop disease symptoms and survived the challenge.

3.2. Neutralization of lethal toxin in vitro

The ability of hp-mAb^{PA} and pp-mAb^{PA} to neutralize toxin activity in vitro was examined. pp-mAb^{PA}, when mixed with PA prior to exposure to murine macrophages, was able to neutralize toxin activity in a similar and dose-dependent manner (Fig. 2) to hp-mAb^{PA}. Cell viability studies indicated that at 50 ng/ml pp-mAb^{PA} could efficiently neutralize PA resulting in 100% cell survival.

3.3. Animal protection studies

The ability of the pp-mAb^{PA} to protect A/J mice against an injected lethal challenge of *B. anthracis* spores was examined and compared with hp-mAb^{PA}. While all the control mice ($n = 8$) died within 3 days of challenge, the pp-mAb^{PA} ($n = 5$) and hp-mAb^{PA} ($n = 8$) treatment groups remained healthy until the end of the experiment (Fig. 3).

4. Discussion

There is substantial data in the literature to show that anthrax toxin-neutralizing antibodies can confer protection across a range of animal species, including primates, against aerosol challenge with spores of *B. anthracis* when administered prior to or immediately post challenge [6–8]. The ability to treat exposed individuals several days after a covert bio-terror attack would result in a significant reduction in mortality. Given that the half-life of human antibodies is approximately 20 days [10], it raises the possibility that a single dose of antibody may be sufficient to treat an infected individual, a property offering considerable logistic advantages in a mass casualty setting. In the past, the major roadblock to the adoption of this approach for the treatment of humans has been access to high affinity, anthrax toxin-specific antibodies that are non-toxic to humans, possess a significantly long half-life, and that can be produced on a commercial scale. To address these issues, researchers have been attempting to develop humanized or fully human PA-specific toxin neutralizing monoclonal antibodies [15,16].

Our studies show that plant-produced mAb against PA had both in vitro and in vivo toxin-neutralizing activity. In fact, the pp-mAb^{PA} neutralized anthrax toxin with the same efficacy as the hp-mAb^{PA} both in cell assays and in protection assays in mice. The results of this study suggest that a human monoclonal antibody produced in plants with specificity for PA can be successfully employed for prophylactic use.

In summary, the results of this study demonstrate that fully active human monoclonal antibodies with specificity to PA can be successfully produced in plants and can be employed for prophylactic use. The study also confirms that the plant-based approach is a viable alternative for manufacturing large quantities of biologically active molecules.

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